

# Molecular and Cellular Biochemistry

## Peroxisomes proliferation and pharmacological stimulation of autophagy in rat liver: evidence to support that autophagy may remove the "older" peroxisomes

--Manuscript Draft--

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<b>Corresponding Author:</b>	Gabriella Cavallini Universita degli Studi di Pisa Pisa, Toscana ITALY
<b>Corresponding Author Secondary Information:</b>	
<b>Corresponding Author's Institution:</b>	Universita degli Studi di Pisa
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	Gabriella Cavallini
<b>First Author Secondary Information:</b>	
<b>Order of Authors:</b>	Gabriella Cavallini
	Alessio Donati
	Michele Taddei
	Ettore Bergamini
<b>Order of Authors Secondary Information:</b>	
<b>Funding Information:</b>	
<b>Abstract:</b>	<p>Like mitochondria, peroxisomes produce reactive oxygen species (ROS), compounds which have been implicated to play an important role in many degenerative diseases and aging itself, and an exaggerated ROS production might occur in altered or older organelles. Growing evidence shows that autophagy, a required function in cell housekeeping during fasting, can remove damaged macromolecules, organelles, and membranes selectively. Proliferation of peroxisomes can be enhanced in liver cells by pentafluorooptanoic acid (PFOA), which causes a marked increase of the Acyl CoA oxidase (ACOX) activity and no significant change in urate oxidase (UOX) activity. The administration of antilipolytic drugs to fasted animals was shown to intensify autophagy. Here we tested the hypothesis that autophagy may distinguish and remove older from younger peroxisomes in rat liver. Male Sprague-Dawley rats were given PFOA (150 mg/kg body weight) or vehicle. Animals were sacrificed at different times following PFOA administration, and 3 hours after the induction of autophagy with the antilipolytic agent 3,5-dimethyl pyrazole (DMP, 12 mg/kg body weight). The levels of ACOX and UOX activity were measured in the liver tissue. Results showed that autophagy caused a parallel, significant decrease in both enzymes activity in control rats, and that in PFOA treated rats the effects were different and changed with PFOA time administration. Changes are compatible with the hypothesis that newly formed ACOX-rich peroxisomes are resistant to pexophagy and that sensitivity to pexophagy increases with increasing peroxisomal "age". In conclusion, there is indirect evidence supporting the hypothesis that autophagy may recognize and degrade older peroxisomes.</p>

To the Editor

**Molecular and Cellular Biochemistry**

I am glad to submit you the revised manuscript MCBI-D-16-01228 “Peroxisomes proliferation and pharmacological stimulation of autophagy in rat liver: evidence to support that autophagy may remove the “older” peroxisomes” by Alessio Donati, Michele Taddei, Ettore Bergamini and myself.

I hope that this revised manuscript may now meet requirements made by the Reviewers and can be further considered for publication in Molecular and Cellular Biochemistry. Furthermore, I should mention that MCBI-D-16-01228 was a revised version of the original manuscript MCBI-D-00915 (the manuscript had been revised to meet the comment of Reviewer #2 , Reviewer #1 was strongly positive and recommended acceptance and publication without any change; your e-mail date September 27).

Thank you very much in advance for your kind consideration.

Kindest regards

Gabriella Cavallini

We warmly thank reviewers for helpful comments and criticisms.

**Note for reviewing purposes - Our comments/answers in bold italic**

**Revisions and corrections in the text all in red bold**

**Reviewer #1:** Authors investigated the role of autophagy in regulating hepatic peroxisome homeostasis in a rat model. Autophagic removal of excessive peroxisomes in yeast or mammalian cells including liver has been well studied.

**Reference to the first report of induced autophagic degradation of rat liver cell peroxisomes is given (ref. n. 19) together with one of the first reviews on pexophagy (ref. n. 18, Introduction, 4<sup>th</sup> paragraph).**

The novelty of this study is low. However, the idea that autophagy may selectively remove old peroxisome in preference of new peroxisome is interesting.

**We agree with the comment that the novelty of this study is the proposal that pexophagy in mammalian cells may be selective, like the case of mitophagy (ref. n.7,8) and that in both cases older (possibly defective) organelles may be removed and degraded preferentially.**

However, this study is largely descriptive and data were not convincing enough to support the conclusion. There will be very limited advance in our understanding of peroxophagy from this study.

**This is intended to be a preliminary communication, for this reason the paper has been submitted as “ Short communication”. We agree that this experimental model may deserve further exploration, but these results may be an additional step towards understanding mechanisms underlying selective autophagic degradation of partially dysfunctional peroxisomes in mammals, probably the oldest ones. In addition, our in vivo model might indicate a new promissory avenue for community of researchers working on underlying mechanisms of peroxisomes turnover.**

Major concerns:

1. The authors claimed that DMP can induce autophagy in the liver. More biochemical data from the liver such as morphological for autophagosomes or biochemically for autophagy markers such as LC3 and p62 should be provided. **Evidence showing that DMP can induce autophagy in the liver is sound and the underlying endocrine mechanisms have been fully elucidated. References are given (ref. n.7,19,20,32,38).**

**Biochemical data from the liver such as morphological data for autophagosomes at the electron microscopy level (TEM) can be found in Locci Cubeddu et al.( ref. n.19 ). Now reference to this paper is emphasized (Introduction, 4<sup>th</sup> paragraph).**

**Morphological data for autophagosomes and biochemical data for autophagy marker such as LC3 can be found in Donati et al. (ref. n. 32).**

2. In figure 2, the authors showed increased ACOX activity after PFOA treatment in Day 2 and 4. However, In figure 4, no such increase were shown. There was no explanation for such discrepancy.

**In figure 2, we showed the effects of PFOA administration for 2 and 4 days on the content of ACOX) activity. Results were given as percent changes with respect to not-PFOA treatment and the increase of ACOX activity with PFOA time administration was shown.**

**In figure 4, we showed the effect of DMP treatment and PFOA administration. Results were given as percent changes with respect to not-DMP treatment, therefore the columns PFOA 2d and PFOA 4d represented the total effect of PFOA (100/100) respectively. Unit of enzyme activity ( $\mu\text{mol NAD}^+$  reduced/min) in the whole liver were : 9.8 in NO PFOA; 78 in PFOA 2d; 152 in PFOA 4d.**

3. The manuscript was poorly written and many references on peroxophagy were not cited.

**More references on pexophagy are given (ref. n. 9,10,18,,27,30,31,36,37).**

**Reviewer #2:** The authors describe their work on autophagy of peroxisomes in the liver. They found that autophagy might recognize and degrade older peroxisomes. This is an interesting study. Appropriate methodology has been

employed and the conclusions appear to be justified based on the data at hand. I have a few minor points for consideration.

***We thank the Reviewer for her/his positive comment.***

Minor points:

1. Please move section on ethical approval to the methods section.

***Section on ethical approval was moved to the methods section as suggested.***

2. Please combine results and discussion sections together.

***Results and discussion were combined into one section as suggested.***

3. The authors should discuss further the mechanisms of peroxisome turnover in relation to peroxisomal disorders, the role of peroxisomes in age-related diseases and the process of aging.

***The mechanism of peroxisome turnover in relation to peroxisomal disorders, the role of peroxisomes in age-related diseases and the process of aging were discussed further (Results and Discussion, p. 7, last thirteen line; p. 8, lines 1-6).***

4. The authors need to elaborate on the clinical applicability/relevance of the study findings

***The clinical applicability/relevance of the study findings was highlighted (Results and Discussion, p. 7, last line; p. 8, lines 1-6).***

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**Peroxisomes proliferation and pharmacological stimulation of autophagy in rat liver: evidence to support that autophagy may remove the “older” peroxisomes**

Authors: Gabriella Cavallini\*, Alessio Donati, Michele Taddei, Ettore Bergamini

Affiliation: Interdepartmental Research Centre on Biology and Pathology of Aging, University of Pisa, Via Roma 55, I-56126 Pisa, Italy

\*Corresponding Author: Gabriella Cavallini

Interdepartmental Research Centre on Biology and Pathology of Aging Via Roma 55, I-56126 Pisa, Italy. Tel.: +39 0502218569; fax: +39 0502218569; E-mail: [gabriella.cavallini@med.unipi.it](mailto:gabriella.cavallini@med.unipi.it)

## Abstract

Like mitochondria, peroxisomes produce reactive oxygen species (ROS), compounds which have been implicated to play an important role in many degenerative diseases and aging itself, and an exaggerated ROS production might occur in altered or older organelles. Growing evidence shows that autophagy, a required function in cell housekeeping during fasting, can remove damaged macromolecules, organelles, and membranes selectively. Proliferation of peroxisomes can be enhanced in liver cells by pentafluorooptanoic acid (PFOA), which causes a marked increase of the Acyl CoA oxidase (ACOX) activity and no significant change in urate oxidase (UOX) activity. The administration of antilipolytic drugs to fasted animals was shown to intensify autophagy. Here we tested the hypothesis that autophagy may distinguish and remove older from younger peroxisomes in rat liver. Male Sprague-Dawley rats were given PFOA (150 mg/kg body weight) or vehicle. Animals were sacrificed at different times following PFOA administration, and 3 hours after the induction of autophagy with the antilipolytic agent 3,5-dimethyl pyrazole (DMP, 12 mg/kg body weight). The levels of ACOX and UOX activity were measured in the liver tissue. Results showed that autophagy caused a parallel, significant decrease in both enzymes activity in control rats, and that in PFOA treated rats the effects were different and changed with PFOA time administration. Changes are compatible with the hypothesis that newly formed ACOX-rich peroxisomes are resistant to pexophagy and that sensitivity to pexophagy increases with increasing peroxisomal “age”. In conclusion, there is indirect evidence supporting the hypothesis that autophagy may recognize and degrade older peroxisomes.

**Keywords:** Peroxisomes, Autophagy, Pentafluorooptanoic acid, Antilipolytic drugs, Aging, Rat liver

## Introduction

Peroxisomes and mitochondria are the major sites for oxygen utilization and potential causes of ROS generation [1]. These cellular organelles are especially prone to oxidative damage resulting in a vicious circle in which the ROS production and oxidative damage might increase with the increasing number of older organelles in cells [2, 3]. A sign of aging is a rising amount of altered cellular components which may be a consequence of chronological loss of cellular quality control. Autophagy is a cellular process which removes proteins, cytomembranes and organelles and allows a continuous turnover of all cell components [4]. Several types of autophagic systems have been described based on the nature of sequestered material (e.g. mitophagy, pexophagy, aggrephagy, lipophagy etc.) [5], that fulfill two main functions inside cells: acting as an alternative source of energy and participating in cellular quality control [6].

Mitophagy in mammalian cell was proposed to be responsible for targeting and degrading altered mitochondria rich in mtDNA mutations [7, 8]. Increasing lines of evidence suggest that selective pexophagy plays a major role in mediating peroxisomal quality control in yeast species and mammalian cells [9, 10].

Peroxisomes are abundant and dynamic organelles that can rapidly modulate their size number, and enzyme content in response to nutritional and environmental stimuli [11, 12]. It was shown that in mammalian cells newly synthesized peroxisomes arise by a de novo pathway from the ER, with only a minor fraction being formed by fission of preexisting organelles [13]. Studies in rodents showed a rapidly proliferation and degradation upon the administration and subsequent withdrawal of a variety of xenobiotics, collectively known as peroxisome proliferators [14, 15]. PFOA is a peroxisome proliferator-activated receptor (PPAR) agonist which stimulates proliferation of peroxisomes resulting in a marked increase in ACOX activity with no change in UOX [16]. Therefore, the “new” PFOA-induced peroxisomes might be richer in ACOX than “older” peroxisomes and the induction might cause unbalanced changes in enzyme activity secondary to a transient heterogeneity into the peroxisomal population [17].

In this research, we test the hypothesis that pexophagy may preferentially distinguish and remove older from younger peroxisomes in rat liver by the use of an experimental model consisting in the induction of peroxisome proliferation followed by a timed stimulation of the autophagic degradation of peroxisomes, named pexophagy [18]. This model of autophagic stimulation was proposed and validated with biochemical and morphological techniques [19]. Proliferation of peroxisomes was induced by the administration of PFOA and pexophagy was stimulated in fasted rats by the injection of DMP, an antilipolytic agent, as described by Donati et al. [20]. In view of the time-lag of the peroxisome proliferation after the administration of PFOA [21] and the estimated half-life of liver peroxisomes [22], changes in the ratio of PFOA-inducible acyl CoA oxidase to PFOA-not-inducible urate oxidase enzymes activities were monitored 2 and 4 days following the administration of PFOA, and 3-hours after the injection of DMP.

## Materials and methods

### Materials

All reagents were of analytical and HPLC grade. Solvents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Standard molecules and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q (Millipore-Lab, Bedford, MA, USA) purified water was used for all analyses.

### Animals

Male Sprague-Dawley rats of 2-month of age were supplied by the Pisa University Interdepartmental Research Centre on Biology and Pathology of Aging vivarium. All of the experiments were conducted following the Official Italian Regulation n. 116/92 for the care and use of laboratory animals. All handling and management procedures were approved by the Independent Ethics Committee of the University of Pisa (Approval number: 2A/42155). Animals were kept in a controlled environment (22 °C, 12/12 h light/dark cycle), fed with a standard laboratory diet (Teklad, Harlan, Italy), and had free access to water. Rats were treated intragastrically with PFOA at 150 mg/kg body weight in 0.5 mL corn oil, or with an equal volume of vehicle alone, 48 or 96 hours before sacrifice. Autophagy was induced in overnight fasted rats by an intraperitoneal injection of DMP at 12 mg/Kg body weight in 0.2 mL saline 3 hours before sacrifice. Controls were injected with vehicle alone.

At designated time-points, rats were sacrificed under pentobarbital anesthesia (50 mg/kg body weight, i.p.) and blood samples were collected from the posterior vena cava into test tube containing 0.25 M EDTA. Subsequently, livers were perfused with ice-cold saline via the portal vein to remove blood, surgically isolated, snap frozen in liquid nitrogen, and stored at – 80 °C until analyzed.

### High-pressure liquid chromatography (HPLC) assay of plasma valine

The stimulatory effect of DMP on the rate of liver autophagic proteolysis was monitored by assaying the levels of plasma valine by HPLC as described in [20]. Amino acid separation was carried out on a 4.6 x 250 mm Bio-Sil ODS-5S column (particle size, 5 mm) in a Beckman HPLC system (equipped with 32 Karat software). Valine was determined by measuring the fluorescence of its dansylated derivative with a Jasco spectrofluorometer (340 nm excitation, 525 emission). Norvaline was added as an internal standard to all samples. Results are given as nmol valine/mL plasma.



### Assay of acyl CoA oxidase and urate oxidase

Tissue samples were homogenized (20%, w/v) in ice-cold 0.25 M sucrose. Acyl-CoA oxidase activity was assayed by measuring the increase in absorbance at 340 nm resulting from cyanide-insensitive palmitoyl-CoA dependent NAD<sup>+</sup> reduction as described in [23] with some modifications as described in [24]. Results are given as unit of enzyme activity ( $\mu\text{mol NAD}^+$  reduced/min) in the whole liver. Urate oxidase activity was assayed by measuring the decrease in absorbance at 290 nm resulting from the oxidation of uric acid to allantoin as described in [25]. Results are given as unit of enzyme activity ( $\mu\text{mol uric acid disappeared/min}$ ) in the whole liver.

### Statistical analysis

The analysis of variance (ANOVA) test was used to evaluate differences among multiple conditions. If positive, the Tukey test was used to test for their statistical significance. Values of  $p < 0.05$  were considered to be statistically significant.

## Results and Discussion

Both mitochondria and peroxisomes are recognized and degraded by selective processes of autophagy named mitophagy and pexophagy respectively [26, 27]. It was shown that mitophagy specifically targets and removes the more severely damaged mitochondria, probably the oldest ones [28, 29].

In this research we tested the hypothesis that autophagy/pexophagy too may distinguish and remove older from younger peroxisomes in rat liver by the use of an experimental model consisting in the induction of peroxisome proliferation followed by timely pharmacological stimulation of autophagy. To our knowledge, no previous report can be found, indicating specificity for older peroxisomes of the autophagic machinery, which is usually reported to eliminate redundant organelles [30, 31].

As expected, PFOA administration induced a progressive increase in the liver/body weight ratio (2 days: + 30 %, 4 days: + 70 %, Fig. 1) and caused dramatic changes in peroxisomal enzyme activities compatible with a transient heterogeneity into the peroxisomal population, with an enrichment in “younger” ACOX-rich as compared to “older” relatively ACOX-poor peroxisomes [16, 17]. The effects of PFOA administration on UOX and ACOX activities exhibited different temporal patterns: total UOX activity in the liver showed small changes, while ACOX activity increased dramatically (eight-and fifteen-fold, after 2 and 4 days respectively, Fig. 2) . A previous study had shown that the maximum increase in the concentration of peroxisomal  $\beta$ -oxidation activity in rat liver is achieved within 48 hours after PFOA treatment and that the maximum accumulation of ACOX activity in the whole liver can be expected by day 3 [21].

The stimulatory effect of the administration of DMP on autophagic proteolysis was monitored by studying the changes in the plasma levels of valine [32], and was similar in control and PFOA-treated rats (Fig. 3). As expected, treatment had very rapid effects on the peroxisomal population both in control and in PFOA treated rats. In control (NO PFOA) animals, the decreases in UOX and ACOX activity (30% and 25%, respectively) after treatment with DMP were similar in agreement with previous observation [19], but in PFOA-treated rats the effects were different and changed with the given enzyme and the time after PFOA administration. In fact, 2 days after PFOA treatment, the DMP-induced stimulation of pexophagy caused a 30 % decrease in UOX activity similar to that in NO PFOA rats, but no significant changes in ACOX activity; whereas the same treatment in 4 days PFOA given rats caused minor, not significant, changes in UOX activity and a marked decrease (30%) in ACOX activity (Fig. 4). The ACOX/UOX activity ratio highlights the differences in the effect of DMP injection after PFOA treatment on peroxisomal enzyme activities likely to reflect changes in the composition of the peroxisomal population prone to degradation: in PFOA-treated rats, DMP-induced pexophagy caused a slight increase in the ACOX/UOX ratio by day 2, a highly significant decrease in the ratio

by day 4 (Fig. 5). It may be worthwhile to keep in mind here that the maximum recorded decrease in peroxisome-enzyme activities was about 30%, both in control and PFOA treated rats, and that Huybrechts et al. have shown that peroxisomes in cultured mammalian cells have a half-life of approximately 2 days and a daily fractional peroxisomal turnover rate of approximately 30% [33]. Furthermore, it was shown that autophagy pathway(s) may play a major role in the degradation of recombinant rat UOX, and that the entire organelle rather than a single protein within the peroxisomes may be degraded [34].

In view of the facts that number, size, and enzyme content of peroxisome may vary dramatically in response to changes in cellular demands or after the administration of peroxisome proliferators [11, 15]; that the administration of PFOA triggers peroxisome proliferation and induces peroxisomal beta-oxidation in rats [21] and mice [35] in less than one day but may not induce urate oxidase or catalase; that rats raised on a controlled and highly fixed environment (like NO PFOA animals) exhibit a homogenous population of peroxisomes, while a chemical proliferator can generate a marked polydispersity of peroxisomes [17] it is conceivable that the differences reported here might be secondary to time-related changes in older peroxisomes to be degraded, that could be recognized by the pexophagy machinery.

Evidence is accumulating that damaged peroxisomes are subject to rapid autophagic degradation after that one or more proteins are exposed at the surface of these organelles [36, 37]. Perhaps an increase in ACOX activity without any parallel increase in catalase activity might expose organelles to a higher oxidative injury. In this perspective and in line with the case of mitophagy [7], our data might invite the speculation that pexophagy can recognize “older”, altered organelles to preserve suitable quality.

Finally, since treatment with antilipolytic agent can boost the antiaging effects of caloric restriction [38] by intensifying the physiological response to fasting (i.e. the decrease in blood glucose and insulin and the increase in glucagon and corticosteroids plasma levels [32]), there is support to the hypothesis that accumulation of “older” possibly defective organelles in older cells might take part in the process of aging. Thus, in line with the case of mitophagy, it is conceivable that also a precise removal of aged peroxisomes by pexophagy is required for the maintenance of cellular homeostasis [39]. Further studies are undoubtedly needed to understand how the older peroxisomes communicate with the autophagic machinery in order to promote peroxisomal quality control into physiological cellular processes. Perhaps, the longer is the organelle’s life and the exposure of membrane proteins to the generated ROS, the higher is the level of altered organelle’s proteins to be ubiquitinated and then detected by the autophagosome membrane as suggested by Schrader et al. [40].

A recent study highlights the role of redox communication between peroxisomes and mitochondria into cellular communication networks during cellular and organismal aging [41]. This interplay may be involved into the etiopathogenesis of peroxisomal disorders, like X-linked adrenoleukodystrophy [42]. In this perspective, since treatment

with antilipolytic during fasting was shown to induce autophagy and be beneficial in humans [43, 44], perhaps it might deserve to be tested on the innate and acquired peroxisomal defects that can result in a large variety of human peroxisomal disorders [45].

In conclusion, the safe pharmacological stimulation of pexophagy by antilipolytic agents might be exploited to understand the mechanisms of peroxisome turnover, both in health, aging and diseases like human peroxisomal or age-related disorders [46, 47].

**Conflict of Interest**

The authors declare that they have no conflict of interest.

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## Figure captions

**Fig. 1** Effects of PFOA administration for 2 and 4 days on liver/body weight ratio. Results are given as percent changes with respect to not-PFOA treatment. Results represent the mean  $\pm$  SEM of five cases. Value in NO PFOA was:  $2.86 \pm 0.03$ . ANOVA statistical analysis showed that effects of PFOA administration were significant ( $p < 0.01$ ). Post-ANOVA Tukey test ( $p < 0.05$ ): \*versus 0 days; °versus 2 days

**Fig. 2** Effects of PFOA administration for 2 and 4 days on the content of urate oxidase activity (UOX) and Acyl-CoA oxidase activity (ACOX) in the whole rat liver. Results are given as percent changes with respect to not-PFOA treatment. Results represent the mean  $\pm$  SEM of five cases. Values in NO PFOA were: UOX  $19.5 \pm 0.3$   $\mu$ mol uric acid disappeared/min; ACOX  $9.8 \pm 0.4$   $\mu$ mol NAD<sup>+</sup> reduced/min. ANOVA statistical analysis showed that effects of PFOA administration on ACOX activity were significant ( $p < 0.01$ ). Post-ANOVA Tukey test ( $p < 0.05$ ): \*versus 0 days; °versus 2 days

**Fig. 3** Effects of DMP injection and PFOA administration for 2 and 4 days on plasma levels of valine. Results are given as nmol valine/mL plasma. Results represent the mean  $\pm$  SEM of five cases. Two-way ANOVA statistical analysis (DMP x PFOA): DMP main effect: ( $p < 0.01$ ); PFOA main effect ( $p < 0.05$ ); DMP by PFOA interaction: N.S. Post-ANOVA Tukey test ( $p < 0.05$ ): DMP - versus DMP +

**Fig. 4** Effects of DMP injection and PFOA administration for 2 and 4 days on the content of urate oxidase activity (UOX) and Acyl-CoA oxidase activity (ACOX) in the whole rat liver. Results are given as percent changes with respect to not-DMP treatment. Results represent the mean  $\pm$  SEM of five cases. Two-way ANOVA statistical analysis (DMP x PFOA): UOX: DMP main effect: ( $p < 0.01$ ); PFOA main effect ( $p < 0.01$ ); DMP by PFOA interaction ( $p < 0.01$ ). Post-ANOVA Tukey test ( $p < 0.05$ ): DMP - versus DMP +  
ACOX: DMP main effect: ( $p < 0.01$ ); PFOA main effect ( $p < 0.01$ ); DMP by PFOA interaction ( $p < 0.01$ ). Post-ANOVA Tukey test ( $p < 0.05$ ): DMP - versus DMP +

**Fig. 5** Effects of DMP injection and PFOA administration for 2 and 4 days on the of Acyl-CoA oxidase (ACOX)/urate oxidase (UOX) activity ratio in the whole rat liver. Results represent the mean  $\pm$  SEM of five cases. Two-way ANOVA statistical analysis (DMP x PFOA): DMP main effect: ( $p < 0.01$ ); PFOA main effect ( $p < 0.01$ ); DMP by PFOA interaction: ( $p < 0.01$ ). Post-ANOVA Tukey test ( $p < 0.05$ ): DMP - versus DMP +; \*versus 0 days; °versus 2 days

Fig. 1

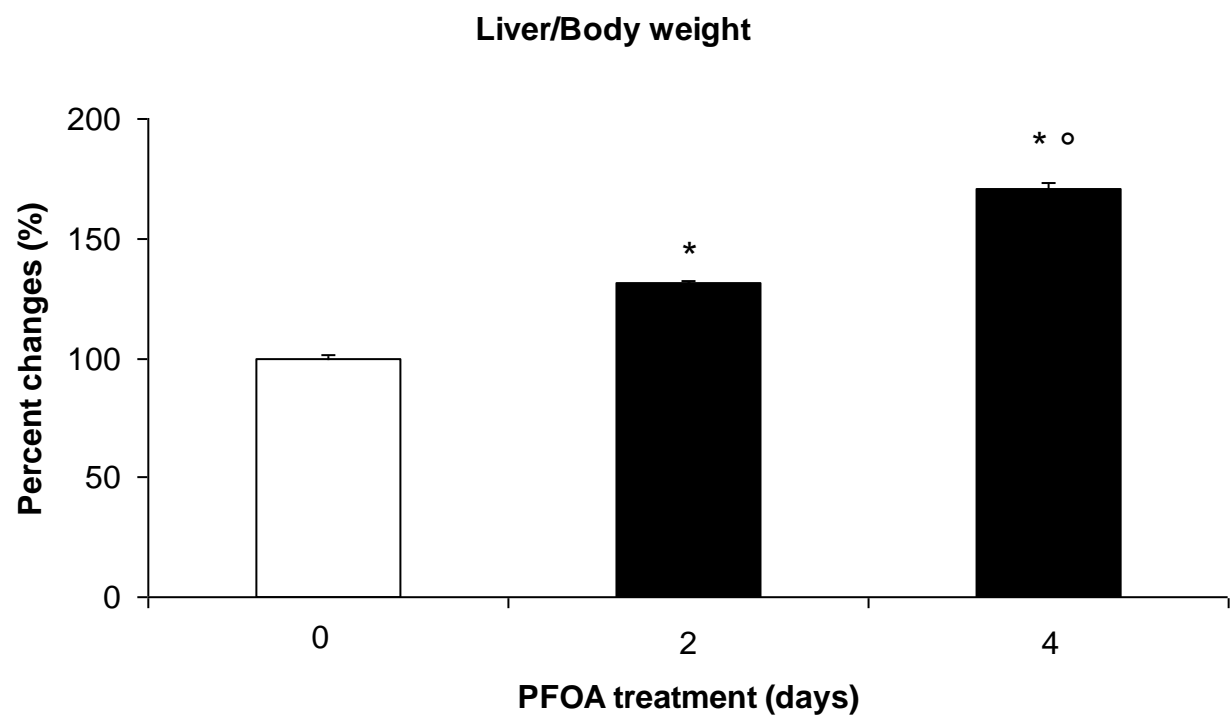


Fig. 2

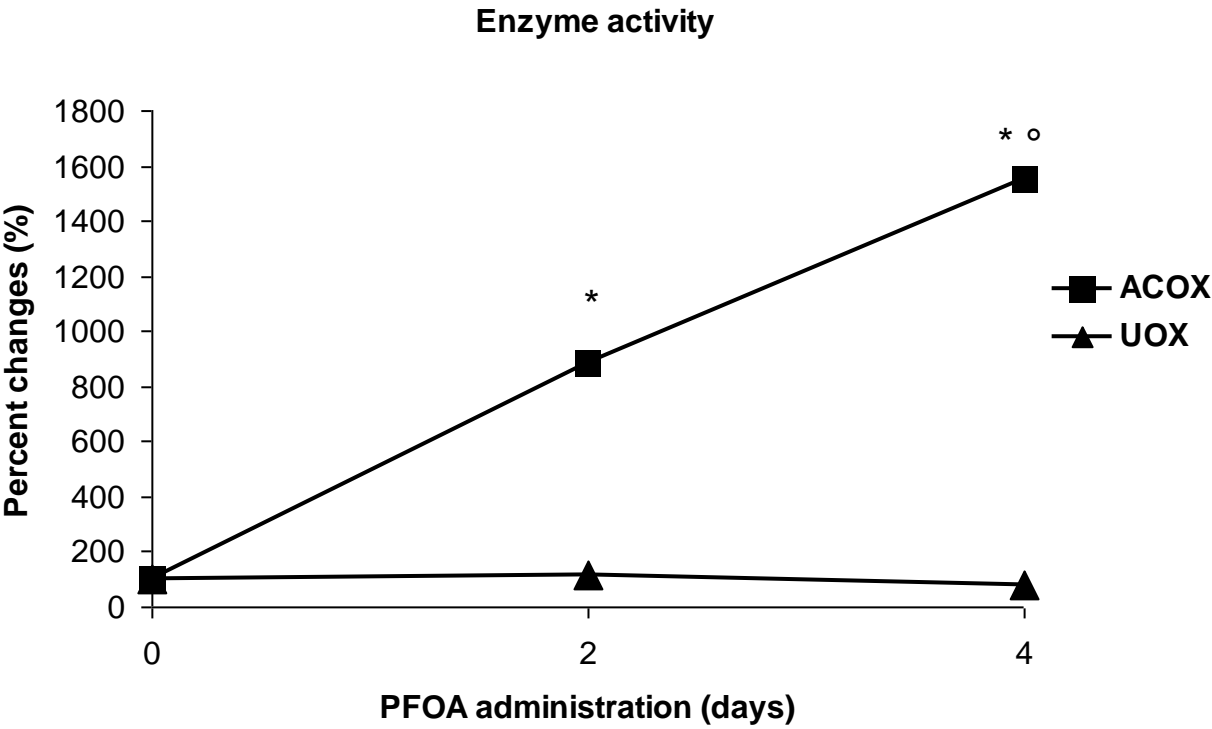


Fig. 3

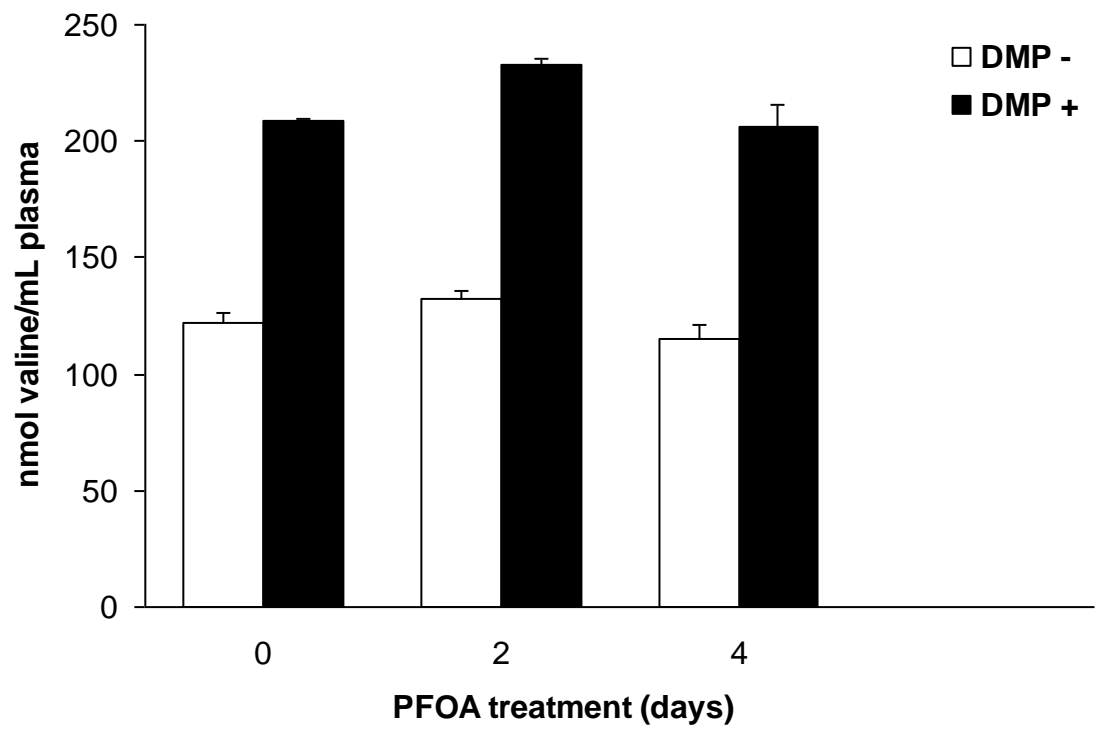


Fig. 4

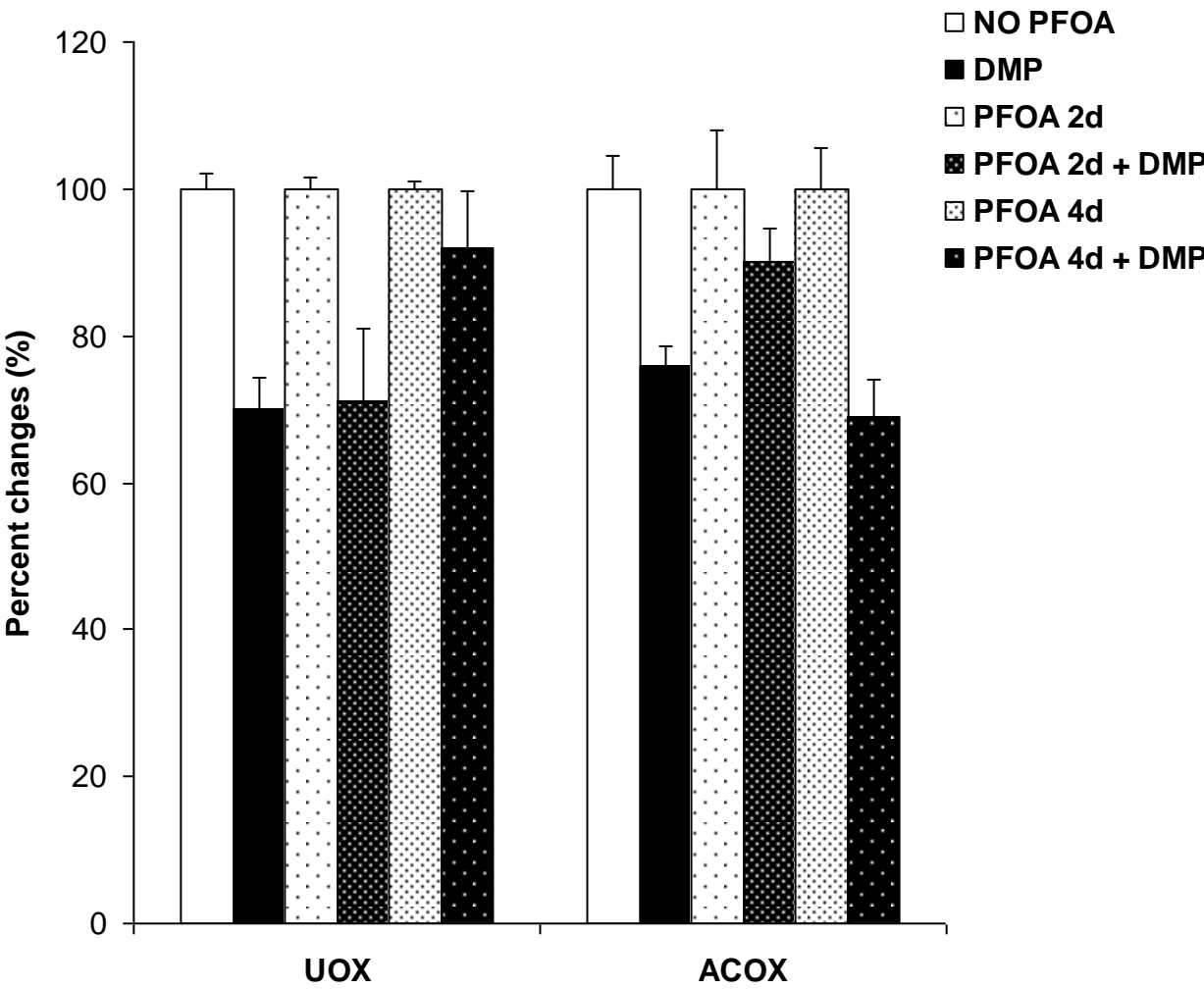


Fig. 5

